Back and forth Wolbachia transfers reveal efficient strains to control spotted wing drosophila populations

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Abstract

1. Since its recent invasion of the European and American continents, the spotted wing Drosophila, *Drosophila suzukii*, has become a burden of the fruit industry. Armed with a highly sclerotized ovipositor, females can lay eggs in a wider variety of ripening and healthy fruits than other *Drosophila* species. Economic losses due to *Drosophila suzukii* reach millions of dollars annually and methods to control natural populations in the field mainly rely on the use of chemical pesticides.

2. We tested if *Wolbachia* bacteria represents a potential ally to control this pest. These symbionts are naturally present in many insects and often induce a form of conditional sterility called Cytoplasmic Incompatibility (CI): the offspring of infected males die, unless the eggs are rescued by the compatible infection, inherited from the mother that protects the embryo. A long-recognised, a strategy called the Incompatible Insect Technique (IIT) makes use of the CI phenotype to control insect populations through the mass release of infected males. To implement this technique in *D. suzukii,* we used back and forth *Wolbachia* transfers between *D. suzukii* and *D. simulans* to identify *Wolbachia* strains that can sterilize *D. suzukii* females despite the presence of *w*Suz, a natural *Wolbachia* infection in this species.

3. We identified two *Wolbachia* strains as potential candidates for developing IIT in *D. suzukii*. Both induce a very high level of CI in this pest which is not attenuated by the presence of *w*Suz in females. Moreover, the newly transferred *Wolbachia* do not affect the fitness or the mating competitiveness of the sterilizing males.

4. *Synthesis and applications*. Although several critical steps still need to be tested and developed outside the laboratory to achieve the control of *Drosophila suzukii* using Incompatible Insect Technique. By an experimental approach in large population cage, we showed that releases of transinfected males limits population size. Thus, we provide in this

study the proof of concept that this technique can be a very promising approach to control *D. suzukii* populations.

Introduction

Drosophila suzukii, the spotted wing Drosophila, has become a major burden for fruit growers since its recent invasion of the European and American continents (Goodhue *et al.* 2011; Calabria *et al.* 2012; Cini, Ioriatti & Anfora 2012; Cini *et al.* 2014; Asplen *et al.* 2015). Although the vast majority of *Drosophila* species are not fruit pests, *D. suzukii* is able to lay eggs on a wide variety of healthy ripening fruits, thanks to a sclerotized ovipositor (Mitsui, Takahashi & Kimura 2006). Internal larval feeding represents a direct damage that can also facilitate secondary infections by pathogens as fungi, yeasts or bacteria (Cini, Ioriatti & Anfora 2012; Hamby *et al.* 2012; Ioriatti *et al.* 2015). Most damages are reported on red fruits, with an approximate $500 million annual loss in the US (Goodhue *et al.* 2011). In France and in Northern Italy, up to 100% destruction was reported on cranberries, strawberries and sweet cherries (Cini, Ioriatti & Anfora 2012). Control of *D. suzukii* populations in the field largely relies on chemical pesticides, a practice with serious drawbacks because of its use close to harvest and the consequent risk of high amount of residues left on fruits. In brief, there is an urgent need for developing effective, specific, and environmentally-friendly methods to fight against *D. suzukii*.

In this context, suppression of pest populations through the mass release of sterilizing males seems relevant, as it is highly specific, not polluting, and does not require the introduction of a new species into the environment (Bourtzis *et al.* 2014; Lees *et al.* 2015). In the Sterile Insect Technique (SIT), males are sterilized by irradiation and released for mating with wild females (Knipling 1955). This approach has already shown its effectiveness against agricultural insect pests like the New World screw-worm fly (Lindquist, Abusowa & Hall

1992). Genetic population suppression approaches, such as Oxitec's RIDL technology could also be considered in principle (Black, Alphey & James 2011), but this technique faces serious regulatory issues in European Union. Finally, one can make use of the bacterial endosymbiont *Wolbachia pipientis* to produce sterilizing males (Boller *et al.* 1976; Riegler & Stauffer 2002), which is the option explored here.

The genus name "*Wolbachia*" designates a highly diverse clade of maternally transmitted intracellular symbionts of arthropods and nematodes, belonging to the α-proteobacteria (Werren 1997). It is well known for its ability to manipulate host reproduction through different strategies that maximize its spread and maintenance in host populations. Among these, the most common appears to be Cytoplasmic Incompatibility (CI), a sperm-egg incompatibility occurring in crosses between infected males and uninfected females or between males and females infected by incompatible *Wolbachia* strains, leading to the death of embryos (Werren 1997). CI can be understood as resulting from a modification/rescue system (*mod*/*resc*) where the *mod* function modifies the sperm during spermatogenesis and the *resc* function, expressed in the egg, rescues the embryo through an interaction with the modified sperm. Recent findings indicate that a pair of genes located in a *Wolbachia* prophage operon, including a deubiquitylating enzyme, are very likely to encode the *mod* and *resc* functions (Beckmann, Ronau & Hochstrasser 2017; LePage *et al.* 2017). Depending on *Wolbachia* and host factors, some strains can both induce and rescue CI, while others express only the *resc* function, meaning that they do not induce CI but can rescue the embryos against the *mod* function expressed by other *Wolbachia* strains (Poinsot *et al.* 1998). CI has led to the proposal and development of the Incompatible Insect Technique (IIT), analogous to the SIT, where sterilization of the targeted populations is achieved by the release of *Wolbachia*-infected males incompatible with the resident females (Boller *et al.* 1976; Riegler & Stauffer 2002). The first successful application of IIT was achieved in Burma where the target

population of *Culex pipiens*, vector of the filariasis, was almost eliminated (Laven 1967). Promising results were also obtained more recently in mosquito species under semi-field (Chambers *et al.* 2011; Moretti & Calvitti 2013; Atyame *et al.* 2015) and field (O'Connor *et al.* 2012) conditions. While initially limited to species where natural *Wolbachia* infections allowed the expression of CI, this method came to the fore again within the last decade, thanks to the possibility to transfer *Wolbachia* strains between species (Hughes & Rasgon 2014). This approach has been successfully tested in the agricultural pest *Ceratitis capitata*, naturally uninfected, where the transfer of two *Wolbachia* strains from *Rhagoletis cerasi* led to the expression of a high level of CI (Zabalou *et al.* 2004b).

Here we aim at developing this promising approach in *D. suzukii*, where we face an additional challenge linked to the presence of *w*Suz, a natural *Wolbachia* strain, found in most *D. suzukii* populations but at variable frequencies (Hamm *et al.* 2014; Cattel *et al.* 2016a). Data indicated that *w*Suz is the only natural infection in *D. suzukii* where it does not induce strong CI (Hamm *et al.* 2014; Cattel *et al.* 2016a). These results indicate that the implementation of IIT in *D. suzukii* requires the introduction of a foreign *Wolbachia* strain that would induce a strong CI in *D. suzukii* and, crucially, a CI that would not be rescued by *w*Suz.

To identify such a strain, we first introduced *w*Suz in *Drosophila simulans* where many *Wolbachia* infections are maintained. This allowed us to assess the rescue capacities of *w*Suz against multiple CI-inducing strains after a single trans-infection experiment. We thus selected three promising strains that were then introduced in *D. suzukii* leading to the identification of two strains that induce a nearly complete CI in this background, regardless of the presence of *w*Suz in females. In addition, the transinfected males showed a similar competitiveness compared to naturally infected or uninfected males and are able to induce a high level of CI during all their life. Finally, we demonstrated that in large population cages,

the IIT can be very efficient to limit the increase of *D. suzukii* populations size. We thus obtained *D. suzukii* lines combining the properties required for an effective implementation of IIT.

Materials and methods

MICRO-INJECTIONS AND MATERNAL TRANSMISSION MEASUREMENT

Micro-injections were performed between *D. suzukii* and *D. simulans*, in both directions, using a micro-capillary needle to transfer the cytoplasm of infected embryos into uninfected ones, at the Fly Facility of the Department of Genetics of the University of Cambridge, following Poinsot *et al.* (1998). Adult females emerging from the injected embryos (G0 females) were crossed with uninfected males of the same genetic background (line STCP in *D. simulans* and Fr-BE-Ø in *D. suzukii*) and allowed to lay eggs during 5 days. We checked the presence of *Wolbachia* by PCR (see Table S1 for the protocol) in all G0 females and kept the offspring of the infected ones. This process was repeated until a perfect maternal transmission of *Wolbachia* was observed. All injected lines were maintained in the lab for at least 8 generations before beginning experiments.

WOLBACHIA STRAINS, DROSOPHILA LINES AND REARING PROCEDURES

Crossing experiments in *D. simulans* involved 10 *Wolbachia* strains, all belonging to the supergroup A (Martinez *et al.* 2015), 9 of which have been used in earlier studies (Poinsot *et al.* 1998; Zabalou *et al.* 2008; Veneti *et al.* 2012; Martinez *et al.* 2014) (see Table S2).

In *D. suzukii*, we used one isofemale line, named Fr-BE-Ø, naturally free of *Wolbachia*, collected in 2012 in Bellegarde (France) and maintained since as a mass population. A line infected by *w*Suz (Fr-BE-*w*Suz) was obtained by back-crosses (see Cattel

157 *et al.* 2016b). The Fr-BE-Ø and Fr-BE-*w*Suz lines were maintained for respectively ~50 and
158 40 generations before the crossing experiments. The Fr-BE-Ø line was also the recipient for
159 injections of *Wolbachia* strains from *D. simulans*.
160 *D. simulans* and *D. suzukii* lines were reared on a cornmeal diet (agar: 1%, dextrose:
161 8.75%, maize: 8.75%, yeast: 2%, nipagin: 3%) and maintained in an incubator at constant
162 temperature (22°C) and humidity (50%) with a 12-hours light/dark cycle.
163
164 CROSSING EXPERIMENTS
165 To analyze the *resc* function of *w*Suz in *D. simulans* we first performed mass crosses using
166 infected females carrying *w*Suz with males infected by each of the *Wolbachia* strains
167 available (see Table S2). Freshly emerged adults were sexed and placed separately into
168 cornmeal diet tubes to ensure the virginity of flies. Ten virgin males (3 to 5-days old) and ten
169 virgin females (5 to 6-days old) were allowed to mate in food vials for 24h. Females were
170 then allowed to oviposit for 48h on grape-juice agar in a petri dish. The total number of
171 hatched and unhatched eggs was recorded 48h after removal of the females. Six such mass
172 crosses were performed for each *Wolbachia* strain tested.
173 Based on the results of mass crosses, three candidate strains were identified (*w*Ri,
174 *w*Tei and *w*Ha). To quantify more precisely the CI relationships between *w*Suz and these
175 candidate *Wolbachia* strains, individual crosses were performed in *D. simulans* following the
176 same protocol except that we used only 3-days old virgin males and 5-days old virgin females
177 and that the egg hatch rates were estimated individually.
178 In *D. suzukii*, in order to make sure that mating had taken place, mating was either
179 observed, confirmed by the hatching of at least one egg, or by the presence of sperm in the
180 spermathecae. We also confirmed *D. suzukii* female's virginity before mating by placing them
181 individually in a petri dish for egg-laying during 48h. Only 13 of the 70 females tested laid

eggs, but none of them hatched, confirming their virginity. For all individual crosses, at least 20 repetitions were obtained, excluding females that laid fewer than 10 eggs. To account for variation in background embryonic mortality (not related to CI), we used a corrected index of CI (CI_{corr}) (Poinsot *et al.* 1998) calculated as follows : $CI_{corr} = [(CI_{obs} – CCM)/(100 – CCM)] * 100$, where CI_{obs} is the percentage of unhatched eggs observed in a given incompatible cross, and CCM is the mean mortality observed in the control crosses.

MEASURE OF LIFE HISTORY TRAITS

Wolbachia infection can negatively affect the fitness of its host depending on the *Wolbachia* strain, the host genotype and the environmental conditions. Because such costs could undermine the effectiveness of the IIT, we measured different life history traits on the lines transinfected by *w*Ha and *w*Tei and compared them to the naturally infected and uninfected lines (Fr-BE-Ø and Fr-BE-*w*Suz). The larval development conditions were standardized in these experiments by depositing 50 eggs of each line in 2mL of cornmeal diet placed in a tube with agar and sugar (10%). We prepared at least 12 such tubes per line and used the freshly emerged adults for the different measures.

We first measured the survival of the pre-adult stages, that is, from egg to adult. In each tube where 50 eggs were deposited, we counted the number of adults that emerged. We then measured the adult survival for both sexes. For each line, 10 freshly emerged adults were placed into a tube containing sweetened 10% agar (10 replicates per sex) and the mortality was recorded every day. Finally, to measure fecundity and hatch rate, we placed 10 virgin males and 10 females (1-day old) in a cornmeal diet tube during 48h for mating. Females were then allowed to oviposit for 48h on grape-juice agar in a petri dish. The total number of eggs and their hatch rate were recorded 48h after removal of the females. At least 18 repetitions were performed for each line.

THE EFFET OF MALE AGE ON CI INTENSITY

To quantify the effect of ageing on CI levels in *D. suzukii* transinfected lines, we used the same protocol as described above for the mass crosses experiments in *D. simulans*. The effect of male age on CI intensity was tested for three ages, 3-4, 7-8 or 11-12 days (6 replicates per age), by crossing males infected by *w*Ha or *w*Tei with females naturally infected or not by *w*Suz. Control crosses were performed (between uninfected males and females, and between males and females carrying *w*Suz) in order to assess the effect of male age on hatch rate regardless of CI. Here again a corrected CI index (CI_{corr}) was used in our analysis.

MATING COMPETITIVENESS OF TRANSINFECTED MALES

The mating competitiveness was tested for males infected by *w*Ha by mixing 40 virgin females with different ratios of these sterilizing males with males carrying *w*Suz or uninfected males in cages of 30×30×30 cm. Five ratios were tested (4 replicates per ratio): 1:1 (20♂ø:20♂*w*Ha:40♀ø), 1:5 (7♂ø:35♂*w*Ha:40♀ø), 1:10 (4♂ø:40♂*w*Ha:40♀ø) and two control ratios, 1:0 (40♂*w*Ha:40♀ø) and 0:1 (40♂ø:40♀ø). Similarly, the same ratios were used with males and females carrying *w*Suz instead of being uninfected. As for previous experiments, we used 5-6 days-old females and 3-4 days-old males. Females were first placed in the cages followed by the simultaneous release of all males, and mating was allowed for 48h, with food and water supply (two recipients containing 50 ml of cornmeal diet, and two sweetened water sources with 10% sugar). Thereafter, females were allowed to oviposit for 48h on grape-juice agar in petri dishes which were then replaced with new ones for another 48h. The total number of hatched and unhatched eggs was recorded 48h after removal the petri dishes from the cage. We computed the competitiveness index (C) (Fried 1971) to compare the performance of sterilizing and compatible males, which is defined as follows: $C=(N/S)*[(H_c-H_i)/(H_i-H_s)]$, where N is the number of "compatible" males, S is the number of

incompatible males, H_c is the hatch rate in the compatible crosses, H_i the hatch rate observed in the different ratios tested and H_s is the hatch rate in clutches from females exclusively crossed with incompatible males. Similarly, expected hatch rate values in male competition experiments were calculated as follows: $[(N*H_c)+(S*H_i)]/(S+N)$.

PROOF OF CONCEPT OF THE IIT EFFECTIVENESS

The IIT can be used to decrease the population size of the targeted species but also to limit the introduction and the population growth, and this is the point we tested here using males infected by *w*Ha. Two cages of 3x3x2 meters were placed separately in climatic chambers with similar conditions of temperature (22°C), humidity (50%), and light (light/dark cycle of 12-hours). In each cage, we placed 6 bottles of 1L of a cornmeal diet with a red fruits mixture (red fruits: 50%, agar: 2.6%, yeast: 12%, maize flour: 18%, sugar: 17%, nipagin: 0.4%) and 10 bottles of 10cL of sweetened water (10%). In the control cage, 20 4-6 days old mated females and 20 males (half uninfected and half infected by *w*Suz) were introduced at the beginning of the experiment and then again every 7 days. In the second cage, called the "IIT cage", the same protocol was followed except that in addition to the 40 individuals released every 7 days, we introduced simultaneously 260 sterilizing males, corresponding to a ratio of 13:1 (260♂*w*Ha:20♂(10♂ø+10♂*w*Suz)). This experiment lasted 62 days, that is, about 9 weeks, so that 360 individuals were released in the control cage and 2700 in the IIT cage (among which 2340 were sterilizing males). The aim of the experiment was to follow the evolution of the population size over time. Six nesting sites (grape-juice agar in a petri dish of 9 cm) were placed in each cage and renewed every 48h. For each nesting site, the number of eggs laid was used as a proxy of the population size and the hatch rate was determined. At the end of the experiment, all living flies were captured and counted.

STATISTICAL ANALYSIS

We used generalized linear mixed models (GLMM) (binomial family) to analyse all hatch rates data.

- For the mass and individual crosses in *D. simulans* and in *D. suzukii*, the *Wolbachia* strain was included as a fixed factor and the replicates as a random factor.

- For the mating competitiveness analysis, the factors “ratio” and “female’s status” were included as fixed explanatory factors and the replicates as a random factor. Exact binomial tests were then used to compare the observed and expected hatch rates in the mating competitiveness experiment. Survival data were also analyzed by GLMM (gamma distribution and inverse link). The *Wolbachia* infection was included as a fixed explanatory factor and the replicates vials as a random factor.

- Fecundity data was analyzed using a GLMM (poisson family); the *Wolbachia* strain was included as a fixed effect and the replicates as a random factor.

- The survival rate from the egg to adult stage was analyzed with a linear mixed-effects model (Gaussian distribution) were the *Wolbachia* infection was included as fixed explanatory factor and the replicates as a random factor.

- In the last experiment, the number of eggs laid and the hatch rate were analysed using a GLM with a poisson and binomial families respectively.

Analysis were performed in R version 3.3.0 (R Core Team 2016), using the package *lme4* for all mixed models (Bates *et al.* 2014).

Results

*w*Suz INJECTION AND CROSSING EXPERIMENTS IN *D. SIMULANS*

The cytoplasm of *D. suzukii* embryos infected by *w*Suz was injected into 234 *D. simulans* *Wolbachia*-free embryos. Seven isofemale lines proved to be infected by *Wolbachia* in G0.

Patterns of maternal transmission of *w*Suz are presented in Fig. S1. One isofemale line showing a 100% transmission from G2 to G3 (20 individuals tested) was selected for CI tests.

CI experiments were designed to select potential *Wolbachia* strains for the sterilization of *D. suzukii* populations, that is, strains that would induce CI even when females carry *w*Suz. To this end, we performed crosses in *D. simulans* between males infected by candidate strains and females carrying *w*Suz. In control crosses, *i.e.* crosses between males and females both infected by *w*Suz, the hatch rate was 97.5%. Among the 10 *Wolbachia* strains tested, known to induce CI in this genetic background (STCP, Martinez *et al.* 2015), three did not appear to induce CI against *w*Suz-infected females. Indeed, hatch rates were not significantly reduced in crosses involving the *w*Stv, *w*Pro and *w*MelCS strains compared to the control crosses (Fig. 1). This indicates that *w*Suz does express a functional *rescue* in *D. simulans* against these strains, and discards them as potential candidates. On the contrary, reduced hatch rates were observed in crosses involving males carrying the other strains. Following Poinsot *et al.* (1998) we computed a CI index (CI_{corr}) taking into account the basal embryonic mortality, to indicate only the proportion of embryos killed by CI. The highest CI_{corr} levels were observed with *w*Ri (57,99% ±13,73), *w*Ha (82,59% ±11,60) and *w*Tei (84,74% ±11,78) (Fig. 1).

Focusing on these three promising strains, we performed individual crosses between males carrying these strains and females either uninfected (used as control) or infected with *w*Suz to characterize more precisely the rescue capabilities of *w*Suz. We thus showed that the presence of *w*Suz in females partially rescues the CI induced by *w*Ri (with a 29% decrease of CI_{corr}) (z=3.53, P<0.001) and *w*Tei (with a 23% decrease in CI_{corr}) (z=3.57, P<0.001) but not *w*Ha (z=0.09, P=0.97) (Fig. 2). At that stage, *w*Ha thus appeared to be the most promising strain, but we injected all the three strains into *D. suzukii* in case host effects would change the rescue capabilities of *w*Suz in its natural host.

CI EXPRESSION OF *WOLBACHIA* CANDIDATES IN *D. SUZUKII*

The cytoplasm of *D. simulans* embryos infected by *w*Ri, *w*Ha or *w*Tei was injected into uninfected *D. suzukii* embryos. As for *D. simulans*, isofemale lines were created from infected females for 2 more other generations until perfect transmission of *Wolbachia* was observed (from G2 to G3; see details in Table S3). For each *Wolbachia* strain, one isofemale line was then selected for crossing experiments.

We performed CI crosses to assess (*i*) if *w*Ri, *w*Ha and *w*Tei can induce CI in *D. suzukii* (transinfected males crossed with uninfected females) and (*ii*) if *w*Suz in its natural host is able to rescue these effects (transinfected males crossed with females infected by *w*Suz). We found that *w*Ha and *w*Tei induce strong CI when infected males are crossed with uninfected females in *D. suzukii* (95.57% and 96.46% CI_{corr}, respectively), in contrast to *w*Ri for which the percentage of unhatched eggs was nearly as low as in the control compatible crosses (18.21% CI_{corr}) (Fig. 3). In addition, the low CI induced by *w*Ri was fully rescued by the presence of *w*Suz in females, while the strong CI induced by *w*Ha and *w*Tei was not. When males infected by *w*Ha were crossed with females carrying *w*Suz, only 34 eggs hatched in total out of the 960 eggs laid (3.54%). Moreover, no egg hatched in 13 of the 30 individual crosses. For the *w*Tei strain, 33 eggs hatched out of 842 (3.92%) with 0% hatch rates in 25 of the 33 individual crosses. In two crosses, higher hatch rates (94.7% and 54.6 %) were seen, suggesting the induction of CI may occasionally fail or that occasionally some males can be uninfected, although an infection rate of 100% has always been observed in the line used here.

LIFE HISTORY TRAITS OF THE TRANSINFECTED LINES

Adult longevity data revealed a significant effect of the infection status on this trait in both males and females (♀: χ^2= 121.95, d.f=3, P<0.001; ♂: χ^2= 38.98, d.f=3, P<0.001). However, this effect does not indicate any physiological cost of the *w*Ha or the *w*Tei *Wolbachia* strain.

On the contrary, males and females infected by *w*Tei had a greater longevity than the other lines (Fig. S2A and B). In females, there was no significant difference between the three other lines (uninfected, *w*Suz and *w*Ha) (Fig. S2A) while in males, individuals infected by *w*Suz showed a higher longevity than uninfected ones (Fig. S2B).

The infection status also affects the fecundity with a significantly larger number of eggs laid in 48h in the *w*Tei (26.39 ±12.03) and *w*Suz lines (26.22 ±8.17) (between which no significant difference was found; GLMM: z=-0.32, P=0.752). No significant difference was detected between the uninfected **(**21.15 ±10.82) and the *w*Ha (22.29 ±9.14) lines (GLMM: z=-0.74, P=0.456) (Fig. S3A). We also found an effect of *Wolbachia* infection on hatch rates, irrespective of CI (Fig. S3B): the *w*Tei and uninfected lines show higher basal hatch rates (91.33% ±15.59 and 94.59% ±9.14 respectively) than the *w*Ha and *w*Suz lines (83.31% ±25.50 and 83.83% ±25.36).

The infection status also appears to impact survival rates at the pre-adult stage, that is, from egg to adult. Uninfected individuals showed a significantly higher survival rate than the other lines **(**ø: 75.33% ±6.34; *w*Suz: 64.38% ±12.63; *w*Tei: 64.00% ±8.49; *w*Ha: 62.17% ±3.86), while there was no significant difference between the three infected lines (Fig. S4).

MALE AGE AND CI INTENSITY

In the control crosses, although hatch rates varied slightly between experiments performed with young or old males, there was no trend indicating an increase or decrease of basal hatch rates with male age (Fig. 4).

In contrast, we observed an overall decrease in CI intensity with male age (Fig. 4A and B). In crosses between males infected by *w*Ha and uninfected females, the CI_{corr} dropped from 97.65% (±2.81) in 3-4-days-old males to 79.72% (±11.97) in 11-12-days-old males. In

crosses with *w*Suz-infected females, the *w*Ha CI_{corr} dropped from 97.30% (±2.50) to 78.22% (±9.36) (Fig. 4A).

The decrease in CI intensity was larger in crosses involving *w*Tei-infected males, dropping from 94.77% (±5.00) to 60.68% (±16.35) and from 93.22% (±6.02) to 60.71% (±10.78) in crosses with uninfected and *w*Suz females, respectively (Fig. 4B). Notably, these experiments also confirmed that females infected by *w*Suz cannot rescue the CI induced by males infected by *w*Ha or *w*Tei, regardless of male age.

MATING COMPETITIVENESS OF STERILIZING MALES

In this experiment, we selected the *w*Ha strain, one among the two candidates, because its CI effect was less affected by male age (full data is provided in Table S4). We first confirmed that *w*Ha-infected males induced nearly 100% CI in *D. suzukii*, regardless of the presence of *w*Suz in females, with an average hatch rate of 0.40% (±1.74) and 0.44% (±0.56) in crosses with uninfected and *w*Suz females, respectively (Fig. 5A). Accordingly, we observed that hatch rates decrease when the proportion of sterilizing males increases (all ratios tested produce significantly different hatch rates, expect the 1:5 and 1:10 ratios, GLMM: z=0.63, P=0.60; Fig. 5A). No effect of females' infection status on hatch rates was detected (GLMM: z=-0.50, P=0.62). The hatch rates observed in the ratios 1:0 and 0:1 (without transinfected males or only transinfected males, respectively) allowed us to calculate the hatch rate expected under the assumption of a similar mating competitiveness between sterilizing and compatible males. For each ratio tested, the observed and expected hatch rates were very close, with slight significant deviations. In three cases, the C index (Fried 1971) was less than 1 (meaning that the transinfected males are less competitive than the compatible males) but the reverse was observed in 3 cases (Fig. 5B). Overall, these results indicate that uninfected and *w*Suz males have very similar mating capacity.

PROOF OF CONCEPT OF THE IIT EFFECTIVENESS

We finally aimed at assessing if repeated releases of sterilizing males could mitigate the increase of a *D. suzukii* population. We performed experiments in two cages of 3x3x2 meters. In these cages, a small *D. suzukii* population was introduced (20 mated females and 20 males, half uninfected and half infected by *w*Suz) and then again every 7 days. In the control cage, where no *w*Ha-infected males were introduced, the number of eggs laid per time unit (48h) increased substantially during the first 38 days, which corresponds to about two generations (Fig. 6A). The increase continued until the end of the experiment, reaching 1408 eggs laid in 48h. In the IIT cage, where *w*Ha-infected males were regularly introduced, the number of eggs laid per 48h remained low and stable. It was between 0 and 49 per time unit until the 53th days, and slightly increased to reach 308 eggs per time unit at the end of the experiment. The multiple releases of sterilizing males thus allowed to keep the population size 5 times smaller than the control cage and this effect is significant (GLM: z=-60.74, P<0.001). Accordingly, the hatch rate observed in the IIT cage was significantly different to the one obtained in the control cage (GLM: z=-20.14, P<0.001) (Fig. 6B). Among the 380 eggs laid in the IIT cage in the last 48h, only 120 were viable (39%) compared with the 1150 hatched eggs among 1408 in the control cage (82%). At the end of the experiment, that is, after 62 days, all live individuals were caught. We thus counted 2184 individuals in the control cage (55% females), and 664 individuals in the IIT cage (41% females). At the end of the experiment, there were thus 4.3 times less females in the IIT cage than in the control one.

Discussion

This study aimed at identifying strains of *Wolbachia* that could be candidates for controlling *D. suzukii* populations through IIT. We achieved this goal in several steps. We first transferred *w*Suz, the natural infection of *D. suzukii*, into *D. simulans* to test its ability to

406 rescue the CI induced by the many *Wolbachia* strains maintained in *D. simulans*. We thereby
407 selected three *Wolbachia* candidates based on incompatibility with *w*Suz, injected them into
408 *D. suzukii*, and validated two as highly promising for the development of IIT.

409 In the course of these experiments, we confirmed previously described CI patterns,
410 namely a strong host effect affecting compatibility relationships in a strain-specific manner
411 (Reynolds & Hoffmann 2002; Weeks, Tracy Reynolds & Hoffmann 2002). We will first
412 discuss these elements before highlighting critical future developments for the effective
413 implementation of IIT in *D. suzukii*. We found strong variation in CI intensity (*mod* function)
414 depending on host factors: *w*Ri induces only moderate CI in *D. suzukii* although it is well
415 known to induce strong CI in its natural host *D. simulans* (Hoffmann, Turelli & Simmons
416 1986; this study), but also after transfer in *D. melanogaster* (Poinsot *et al.* 1998). On the
417 contrary, *w*Tei does not induce CI in its natural host, *D. teissieri* (Zabalou *et al.* 2004a), but
418 induces a high level of CI in both *D. suzukii* (this study) and *D. simulans* (Martinez *et al.*
419 2015; this study). The *w*Suz infection exhibits the exact same pattern in its natural host and in
420 *D. simulans*: it induces very low albeit significant CI (see Fig. S5A, B; 63.7% hatch rate and
421 64.2% respectively). It is clear from these data that a given host should not be generally
422 considered as permissive or refractory to *Wolbachia* infection and CI, given these strong host
423 genotype-by-*Wolbachia* strain interactions. Moreover, the analysis of the CI relationship
424 between *w*Suz and other *Wolbachia* strains in *D. simulans* and in *D. suzukii* reveals that not
425 only the induction of CI (*mod* function) is host dependent, but also the ability to rescue CI
426 (*resc* function).

427 Beyond these CI-relevant results, the main point of this study was to identify
428 *Wolbachia* strains that could be used as biological control of *D. suzukii* populations through
429 IIT. By performing back and forth *Wolbachia* transfers between *D. suzukii* and *D. simulans*,
430 we identified two candidates, *w*Ha and *w*Tei. These two strains induce a very high level of CI

in *D. suzukii*, which is not attenuated by the presence of *w*Suz in females. A number of additional results further confirm that these transinfected lines can be envisaged to implement the control of *D. suzukii* populations. First, *w*Ha and *w*Tei show a perfect maternal transmission. Second, transinfected males induce a high level of CI throughout their life, despite a reduction with ageing. Finally, transinfected males do not suffer from reduced mating competitiveness or other fitness costs. Accordingly, we showed that the repeated release of sterilizing males can limit the explosion of a *D. suzukii* large cage population. Overall, this study provides, at a laboratory scale, a proof of concept that the IIT approach can be powerful to control *D. suzukii* populations.

An efficient IIT program must rely on efficient methods to avoid the release of fertile females, which could result in population replacement rather than population suppression (Bourtzis *et al.* 2014). In case of accidental release, the newly introduced infection would easily spread across *D. suzukii* populations, since the resident infection induces only very low CI. Notably, the fact that the *w*Ha and *w*Tei strains are mutually incompatible (Zabalou *et al.* 2008) means that one strain might still be used for population control in case of accidental release and invasion of the first strain. To circumvent the difficult sexing step, IIT could be coupled with moderate irradiation (Brelsfoard, St Clair & Dobson 2009; Bourtzis *et al.* 2014; Zhang *et al.* 2015) that would be sufficient to sterilize females without affecting the life history traits and competitiveness of males which would be fully sterile thanks to the *Wolbachia* (Calvitti *et al.* 2012; Bourtzis *et al.* 2014). Finally, IIT relies on the massive production of males which seems achievable for such a small and polyphagous insect, but requires additional developments.

The existence of *D. suzukii* lines transinfected by CI inducing *Wolbachia* strains represents a critical step toward the implementation of IIT. Our results indicate that these lines carry a number of crucial properties that make them usable in practice to limit population size

in large population cages. While additional developments are still needed, we are now much closer to make IIT a credible alternative to pesticides to control *D. suzuk*ii populations.

Author's contributions

JC, SC, FV, PG and LM conceived the project and designed its methodology; JC, TA, KN and DL collected the data; JC analyzed the data; JC, SC, FV, PG and LM led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Data accessibility

Data available from the Dryad Digital Repository. DOI: 10.5061/dryad.4c7kq (Cattel *et al*. 2018).

Supporting Information

Table S1. Primers used in this study

Table S2. Details of all *D. simulans* lines used in this study

Table S3. Data on microinjections and maternal transmission of *Wolbachia* strains in *D. suzukii*

Table S4. Data on mating competitiveness of *w*Ha-transinfected males

Figure S1. Details of transmission rate of *w*Suz in isofemale line of *D. simulans*

Figure S2. Effect of *Wolbachia* infection on *D. suzukii* survival

Figure S3. Effect of *Wolbachia* infection on the fecundity and the hatch rate in *D. suzukii*

Figure S4. Effect of *Wolbachia* infection on the survival rate from the egg to adult stage

Figure S5. CI induced by *w*Suz in *D.* suzukii and *D.* simulans

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Figures

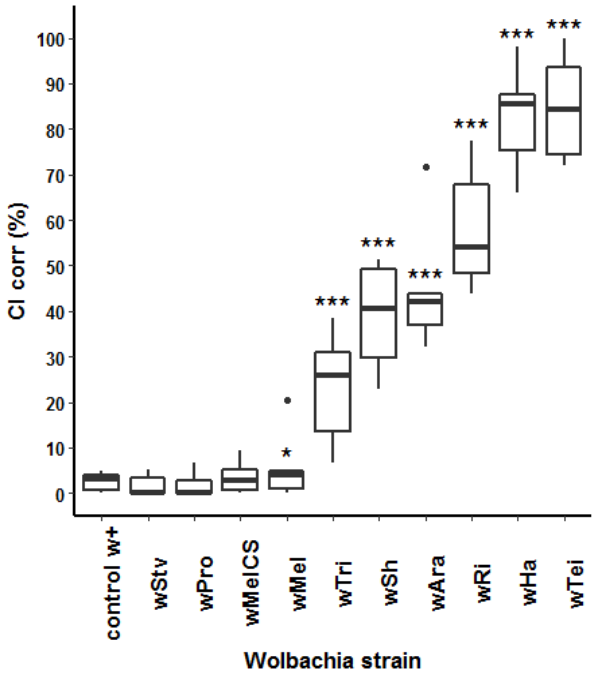

Fig .1. CI levels estimated in mass crosses in *D. simulans* between infected males (*Wolbachia* strains named on the horizontal axis) and *w*Suz-infected females. The CI_{corr} index removes the basal embryonic mortality (estimated in control crosses); it is thus a measure of the CI-related mortality. GLMM (binomial family) was performed for comparisons with the control crosses. ***: $P<0.001$; *: $P<0.05$.

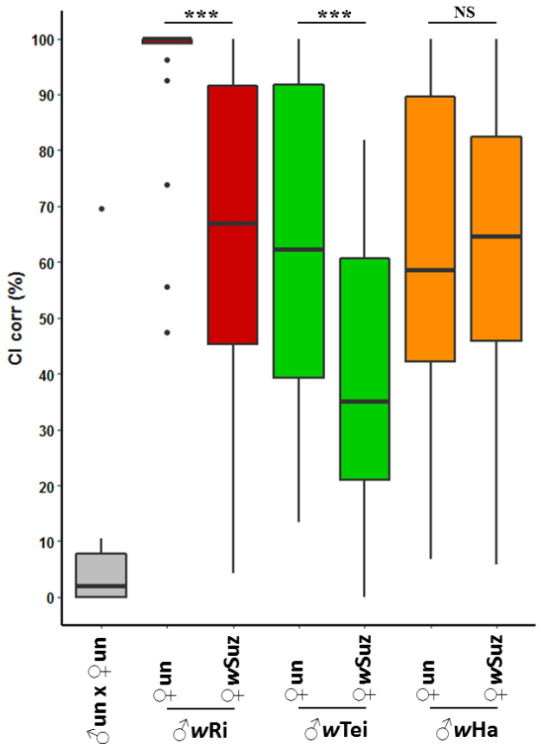

Fig .2. Assessment of the *resc* capabilities of *w*Suz in *D. simulans*. un: uninfected, *w*Suz: infected by *w*Suz, *w*Ri: infected by *w*Ri, *w*Tei: infected by *w*Tei, *w*Ha: infected by *w*Ha. The CI_{corr} index removes the basal embryonic mortality (estimated in control crosses); it is thus a measure of the CI-related mortality. 20 repetitions were performed for each type of cross. ***: $P<0.001$.

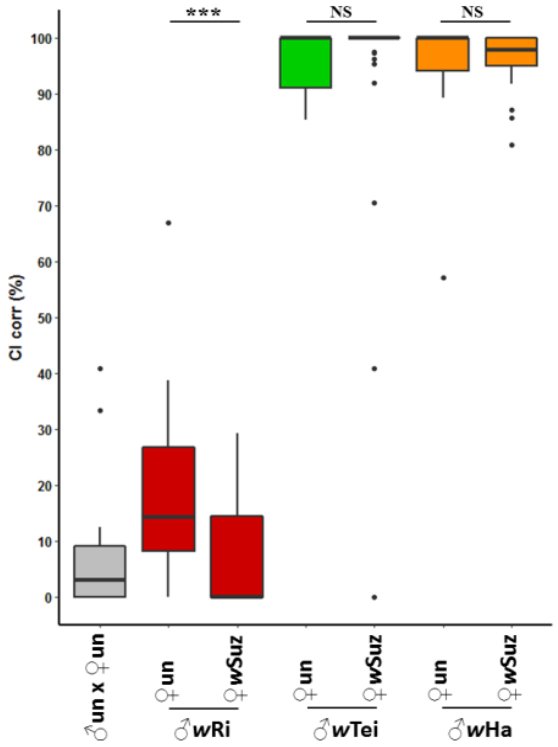

Fig .3. Measure of the *mod* function of *w*Ri, *w*Ha and *w*Tei and the *resc* function of *w*Suz in *D. suzukii*. un: uninfected, *w*Suz: infected by *w*Suz, *w*Ri: infected by *w*Ri, *w*Tei: infected by *w*Tei, *w*Ha: infected by *w*Ha. The CI_{corr} index removes the basal embryonic mortality (estimated in control crosses); it is thus a measure of the CI-related mortality. ***: $P<0.001$.

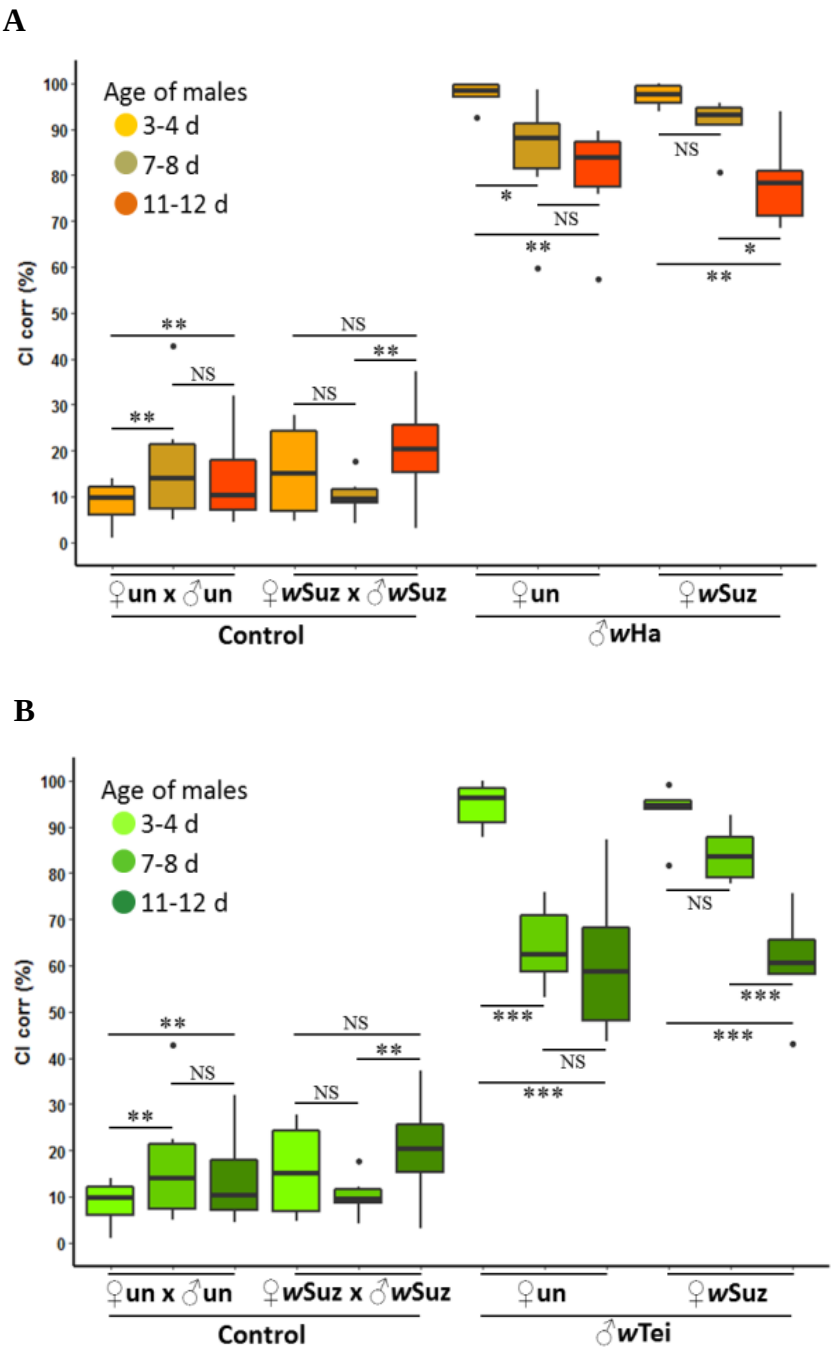

Fig. 4. Effect of male age on CI intensity in *D. suzukii*. For each transinfected line (*w*Ha or *w*Tei), three different ages were tested, 3-4, 7-8 and 11-12 days. A: sterilizing males infected by *w*Ha. B: sterilizing males infected by *w*Tei. un: uninfected, *w*Suz: infected by *w*Suz, *w*Ha: infected by *w*Ha, *w*Tei: infected by *w*Tei. The CI_{corr} index removes the basal embryonic mortality (estimated in control crosses); it is thus a measure of the CI-related mortality. ***:P<0.001, **:P<0.01, *:P<0.05.

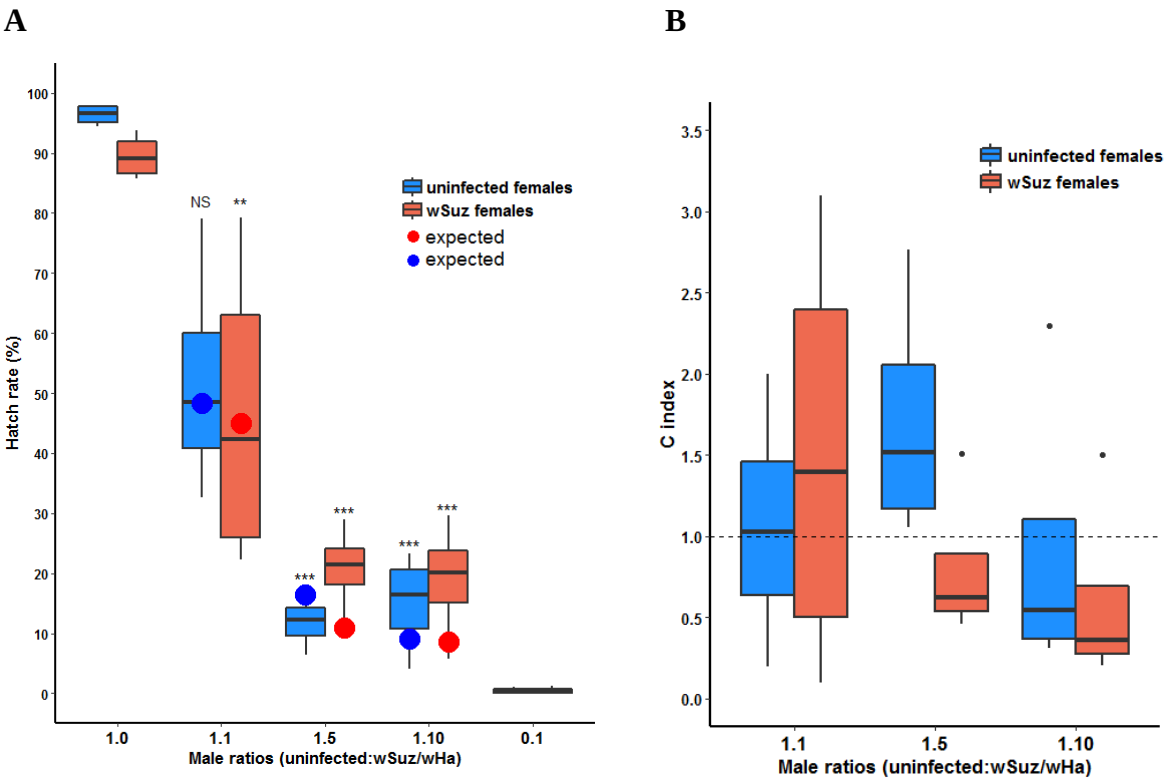

Fig. 5. Mating competitiveness of the *w*Ha-transinfected males. A: observed and expected hatch rates; as expected, hatch rates decrease as the proportion of sterilizing males is increased. Exact binomial tests were performed to compare the observed and expected hatch values. ***: P<0.001; **: P<0.01. B: competitiveness index (C). Both expected hatch rates and C index were computed following Fried (1971) (see Material and Methods for details).

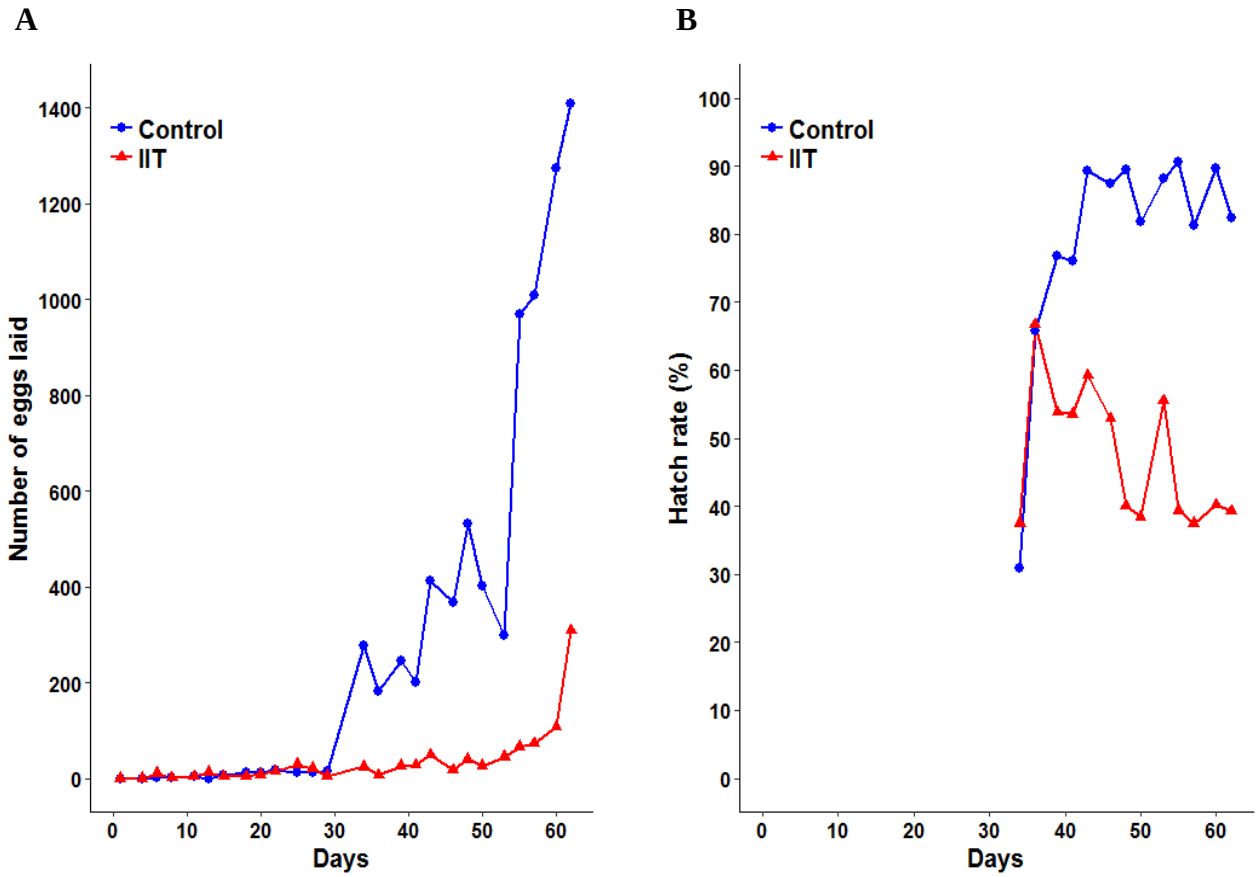

Fig. 6. Evaluation of the IIT effectiveness to limit *D. suzukii* population growth in a large climatic chamber. A: number of eggs laid per 48h at regular intervals. B: hatch rates at regular intervals.